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Protein kinase C inhibits the CAK-CDK2 cyclin-dependent kinase cascade and G1/S cell cycle progression in human diploid fibroblasts

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Abstract

Serum stimulation of human diploid fibroblast IMR-90 cells leads to phosphorylation of $p33^{CDK2}$ at Thr160 and activation of CDK2 kinase, a necessary event for G1/S transition. We report that serum stimulation causes a gradual, sustained increase in the activity of CDK-activating kinase (CAK) that phosphorylates CDK2 at Thr160, which starts by 5 h after serum stimulation and reaches the maximal plateau level at around the G1/S boundary. In this cell type addition of phorbol-12, 13-dibutyrate 5 h but not 16 h after serum stimulation completely inhibits CDK2 kinase activation and DNA synthesis. Phorbol ester treatment does not reduce the protein level of $p33^{CDK2}$, but does inhibit serum-stimulated increases in the CAK activity and CDK2 phosphorylation at Thr160. The suppression of the CAK activity by the phorbol ester is accompanied by decreases in the message levels of both CDK7 and cyclin H, the catalytic and the positive regulatory subunit of CAK, respectively. These results indicate that in IMR-90 cells activation of protein kinase C in the late G1 phase causes cell cycle arrest before the G1/S boundary at least in part through downregulation of CAK and CAK-mediated CDK2 phosphorylation and activation.

Keywords: Protein kinase C; CDK-activating kinase; CDK7; Cyclin H; CDK2; Cell cycle

1. Introduction

Eukaryotic cell cycle is driven by an ordered activation of a family of protein kinases, cyclin-dependent kinases, which are composed of a catalytic subunit CDK and a positive regulatory subunit cyclin [1,2]. Each CDK/cyclin complex is considered to play specialized roles at a defined stage of cell cycle, through phosphorylation of its specific, though largely unknown, substrate proteins [3–6]. Accumulating evidence indicates that CDK2/cyclin A complex mediates initiation and progression of the S phase [5,7–13]. Indeed, CDK2-associated protein kinase activity starts to increase just before the G1/S boundary and continues to rise as cells progress through the S phase [7,8]. It has also been demonstrated that microinjection of antibodies against CDK2 or cyclin A, or antisense cyclin A expression vector prevents serum-stimulated cells from entering into the S phase [9–13]. Activation of CDK2 depends on both assembly with a cyclin molecule (cyclin E in the late G1 phase and cyclin A in the S phase) and phosphorylation of CDK2 at Thr160 [14,15]. Recently, CDK activating kinase (CAK), that phosphorylates CDK2 at Thr160 and activates CDK2, has been identified [16–22]. It has been proved that CAK itself is a CDK/cyclin complex composed of the catalytic subunit CDK7 (homologue of *Xenopus* MO15) [16–19] and a novel cyclin, cyclin H [20,21]. CDK7 (MO15) possesses structural features common to CDK family kinases, including a putative activating phosphorylation site which corresponds to Thr160 of CDK2 [16–19,22]. These recent findings indicate that the CAK-CDK2 kinase cascade plays a pivotal role in cell cycle traverse across the G1/S border.

Protein kinase C (PKC) has been implicated as one of the major regulators of cell growth and proliferation. Earlier studies demonstrated that activators of PKC, including tumor-promoting phorbol esters and diacylglycerol analogues, stimulate DNA synthesis in some cell types [23,24]. It was later shown, however, that under certain circumstances PKC activation rather causes inhibition of cell growth [8,25–27]. We have recently demonstrated in human umbilical vein endothelial cells that the major molec-

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ular target of inhibitory action of PKC is located in the later half of the G1 phase. Thus, the addition of the PKC activator phorbol-12, 13-dibutyrate (PDBu) or 1,2-dioctanoylglycerol in the late G1 phase, but not during the early G1 phase or in the S phase, results in complete inhibition of growth factor-induced CDK2 activation and initiation of DNA synthesis [8]. The effects of the PKC activators are nearly totally abolished in phorbol ester-pretreated, PKC-down-regulated endothelial cells [8]. We have further shown that the PKC activation in the late G1 phase results in complete suppression of growth factor-induced increases in the mRNA level of cyclins A and E, but not CDK2 [8,28]. We have subsequently found that the growth inhibitory mode of PKC action is not confined to endothelial cells but is also observed in human diploid fibroblasts IMR-90. In the present study, to explore in depth the molecular mechanism by which PKC inhibits the activation of CDK2, we examined the regulation by PKC of CAK-mediated phosphorylation of CDK2 at Thr160 in IMR-90 cells. We report here that the PKC activation in the late G1 phase of serum-stimulated IMR-90 cells leads to inhibition of the CAK activity, CDK2 phosphorylation and CDK2 activation. The inhibitory effects mediated by PKC involve suppression of the mRNA levels of both CDK7 and cyclin H. To our knowledge this is the first report describing down-regulation of CAK by a negative cell cycle regulator.

2. Materials and methods

2.1. Cell culture

IMR-90 cells are human diploid fibroblasts derived from fetal lung. They were obtained from the Japanese Cancer Research Resources Bank and maintained at subconfluent state in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (GIBCO) as described previously [29]. Before each experiment cells were grown to confluence and made quiescent by culturing in DMEM containing 0.2% bovine serum albumin (Sigma) (DMEM/BSA) for 48 h.

2.2. Measurement of [^{3}H]thymidine incorporation into DNA

Quiescent cells in 48-well plates were stimulated with 10% fetal calf serum in fresh DMEM and pulse-labeled with [methyl-^{3}H]thymidine (Du Pont-New England Nuclear) (2 μCi/ml) during the last 1 h of various length of incubations. In some wells PDBu (10^{-7} M) (Sigma) was added at indicated time points. Radioactivity incorporated into acid-precipitable material was counted as described previously [29]. For down-regulation of cellular PKC, cells were pretreated with 1 μM of PDBu in DMEM/BSA for 48 h and washed extensively with DMEM/BSA as described [8].

2.3. Measurement of the CDK2 kinase activity and analysis of phosphorylation state of CDK2

CDK2-associated histone H1 kinase activity was measured as described in detail previously, by using a polyclonal antibody raised against the carboxyl terminal sequence of human CDK2 [8]. Phosphorylation state of CDK2 was analyzed as follows. Quiescent cells in 60-mm dishes were incubated in the presence or absence of 10% fetal calf serum and PDBu (10^{-7} M) for 16 h. Then cells were washed twice with phosphate-free Eagle's minimal essential medium and further incubated in the same medium containing [^{32}P]orthophosphate (Du Pont-New England Nuclear) (100 μCi/ml) for 4 h, in the continued presence or absence of 10% dialyzed, phosphate-free fetal calf serum [29] and PDBu. CDK2 was then immunoprecipitated from labeled cells as described before [8], separated on 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P membrane (Millipore). CDK2 was probed with the anti-CDK2 polyclonal antibody and visualized by alkaline phosphatase-conjugated secondary antibody (Zymed) [8]. Radioactivity incorporated into CDK2 was then analyzed by Fuji BAS 2000 Bio-Image Analyzer [8].

2.4. Measurement of the CAK kinase activity

A rabbit polyclonal antibody was raised against a keyhole limpet hemocyanine-conjugated synthetic peptide corresponding to the carboxyl terminal sequence of mouse CDK7 (SGRPVTPPRTANPPKKR) [20]. The antibody specifically recognized mouse and human CDK7 of approx. 40 kDa. CDK7 was immunoprecipitated from cell lysate by the antibody (IgG fraction) according to the method described for immunoprecipitation of CDK2 [8]. The CAK kinase activity of the immunoprecipitate was measured in vitro by using recombinant human CDK2 as a substrate, which was produced in Sf9 insect cells. A full-length human CDK2 cDNA was isolated from cDNA library prepared from SV40-transformed WI-38 cells (VA13, subline 2RA) and ligated downstream of the polyhedrin promoter of pVL1393. Sf9 cells were co-transfected with human CDK2 cDNA-carrying pVL1393 and Baculovirus DNA (BaculoGold, PharMingen) by the lipofectin method. CDK2 protein produced in Sf 9 cells was purfied as described [30]. The phosphorylation reaction was performed at 32°C for 15 min in 25 μl of reaction mixtures containing recombinant human CDK2 (2 μg), 2.5 mM ATP, 4 μCi of [γ-^{32}P]ATP (Du Pont-New England Nuclear), 1 mM dithiothreitol, 10 mM $MgCl_2$ and 25 mM Tris-HCl (pH 7.5). The reaction was terminated by adding 10 μl of 4 × Laemmli's sample buffer, followed by boil-

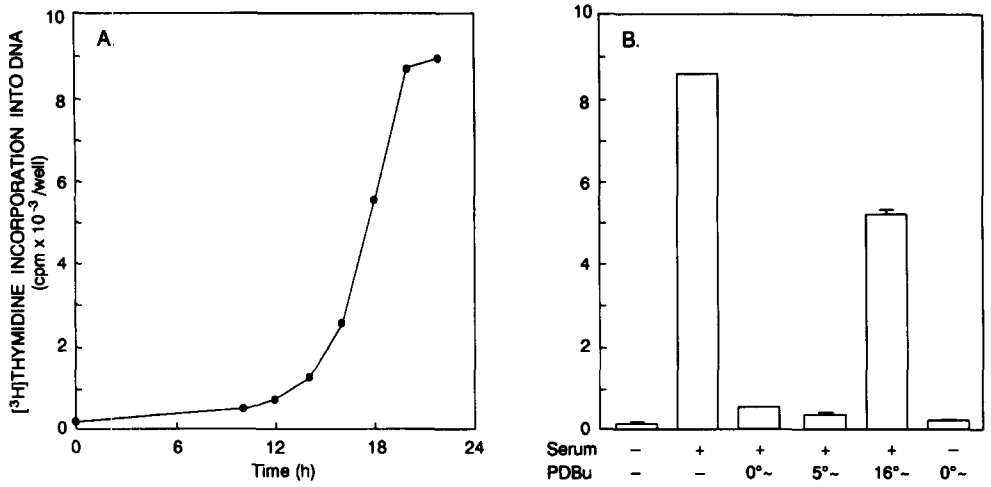

Fig. 1. The effects of serum and PDBu on [^{3}H]thymidine incorporation into DNA of IMR-90 human diploid fibroblasts. (A) Serum stimulation induces the initiation of DNA synthesis after a lag period (G1 phase) of approx. 12 h. Quiescent cells were incubated with 10% fetal calf serum for indicated time periods and pulse-labeled with [^{3}H]thymidine during the last 1 h of incubations. (B) Addition of PDBu either together with (0° ~) or 5 h after (5° ~) serum stimulation nearly completely inhibits serum-induced DNA synthesis. Quiescent cells were incubated with or without serum and PDBu (10^{-7} M) as indicated for 22 h and pulse-labeled with [^{3}H]thymidine during the last 1 h. Data represent the means ± S.E. of three determinations

ing for 5 min. Phosphorylated CDK2 was separated on 12% SDS-PAGE and autoradiographed. Radioactivity incorporated into CDK2 was quantitated by using Fuji BAS 2000 Bio-Image Analyzer.

2.5. Northern blot analysis of CDK7 (MO15), CDK2, cyclins A, E and H

Nothern blot analysis was performed as described previously [8,28]. Ten micrograms of total cellular RNA was prepared by the guanidine isothiocyanate/phenol/chloroform extraction method [31], separated by formaldehyde/1.0% agarose gel electrophoresis and transferred to a nylon membrane (Hybond N)(Amsterdam). The probes for human CDK2, cyclin A and cyclin E were described elsewhere [8,28]. The 560-base fragment (nucleotides 31-590 when A of the initiation codon ATG was numbered as 1) of human CDK7 (MO15) cDNA and the 475-base fragment (nucleotides 19-493) of human cyclin H cDNA were obtained with the polymerase chain reaction amplification method. Each probe was labeled to a specific activity of 2 to 7×10^8 cpm/mg with

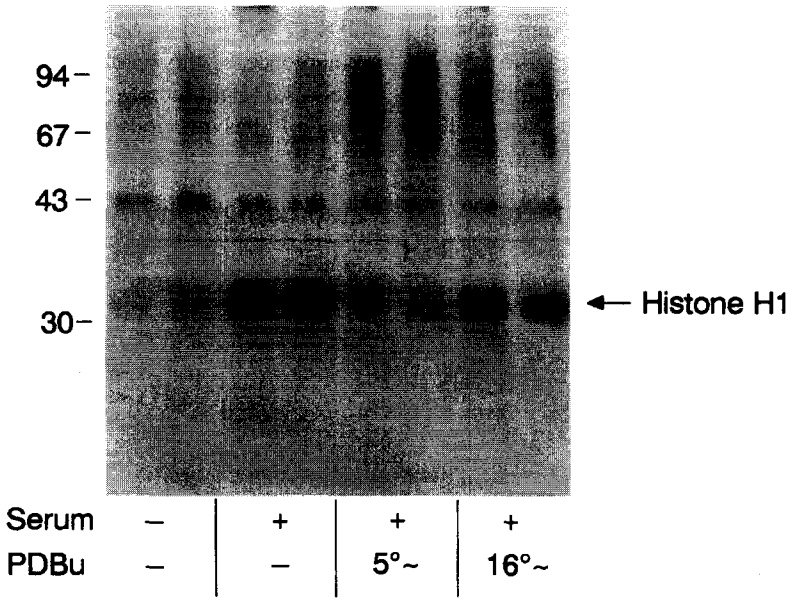

Fig. 2. The addition of PDBu 5 h, but not 16 h, after serum stimulation completely inhibits the activation of CDK2 kinase. Quiescent cells were stimulated with serum and treated with or without PDBu (10^{-7} M) as indicated. After 21 h CDK2 was immunoprecipitated from cell lysate (200 μg of protein) and the CDK2 kinase activity was measured in vitro with histone H1 as a substrate. The position of phosphorylated histone H1 is indicated in the right. Numbers on the left, molecular mass in kDa.

[α-^{32}P]dCTP (Du Pont–New England Nuclear) by the random priming method. Hybridization was performed, membranes washed and autoradiographed as described [8,28]. The radioactivity of corresponding bands was quantitated by the Fuji BAS 2000 Bio-Image analyzer. After stripping radioactive probes off the membrane, the membrane was rehydrized with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, the results being used as an internal control.

3. Results and discussion

Stimulation of quiescent IMR-90 cells with fetal calf serum leads to initiation of DNA synthesis after the G1 phase of approx. 12 h (Fig. 1A). The addition of PDBu (10^{-7} M), either at 0 time point together with serum or 5 h after serum stimulation, similarly results in nearly complete suppression of DNA synthesis measured at 22 h (Fig. 1B). If PDBu is added 16 h after serum stimulation, i.e., after cells have entered the S phase, the inhibitory effect of PDBu is markedly reduced (Fig. 1B). These results indicate that the major target of PDBu action resides in the late G1 phase. Differently from certain types of cells such as Swiss 3T3 fibroblasts [23,24], PDBu does not at all stimulate DNA synthesis in IMR-90 cells, either alone or in combination with serum growth factors (Fig. 1B). The growth inhibitory effect of PDBu is totally abolished in PDBu-pretreated, PKC-down-regulated cells (Table 1), suggesting that the effect of PDBu is mediated by a down-regulation-sensitive isoform(s) of PKC.

PDBu-induced inhibition of DNA synthesis is closely associated with inhibition of CDK2 activation. Thus, as shown in Fig. 2, the addition of PDBu (10^{-7} M) 5 h after serum stimulation completely suppresses serum-induced increase in the CDK2 activity, whereas its addition 16 h after serum stimulation results in only partial inhibition. PDBu does not have any direct effect on the CDK2 activity in vitro when added to kinase assay tubes [8]. As for [^{3}H]thymidine incorporation experiments, the inhibitory effect of PDBu on CDK2 activation is nearly totally abolished in phorbol ester-pretreated, protein kinase C-down-regulated cells. Since it is now established that CDK2 activation is a critical event for the G1/S cell cycle transition [9–13], it is rational to conclude that PKC-mediated suppression of CDK2 activation is, at least in part, responsible for failure of PDBu-treated cells to enter the S phase.

Table 1
PDBu-induced inhibition of DNA synthesis is abolished in PKC-down-regulated cells

	Control	PKC-down-regulated
None	660 ± 40	1220 ± 10
Serum	5770 ± 230	7660 ± 1100
Serum + PDBu	930 ± 70	7800 ± 1000

Data are cpm/well.
Quiescent cells were pretreated with either 1 μM of PDBu (PKC-down-regulated) or 0.1% dimethylsulfoxide (Control) for 48 h, washed extensively, and then incubated in the presence or absence of 10% fetal calf serum and PDBu (10^{-7} M) for 22 h. Cells were labeled with [^{3}H]thymidine during the last 1 h. Data represent means ± S.E. of three determinations.

To study the molecular mechanism by which PKC mediates inhibition of CDK2 activation, we first examined the protein level and the phosphorylation state of CDK2. Shown in Fig. 3 are immunoblots and autoradiograms of CDK2 immunoprecipitated from cells metabolically labeled with [^{32}P]orthophosphate. Cells were serum-stimulated, treated with or without PDBu 6 h later, and then labeled with ^{32}P during the final 4 h of total 20 h incubations. Quiescent cells express a moderate amount of 33 kDa CDK2 protein. Serum stimulation for 20 h leads to an emergence of the second band with a higher mobility, as well as a slight increase in the amount of the major 33 kDa band. It has previously been shown that CAK-mediated

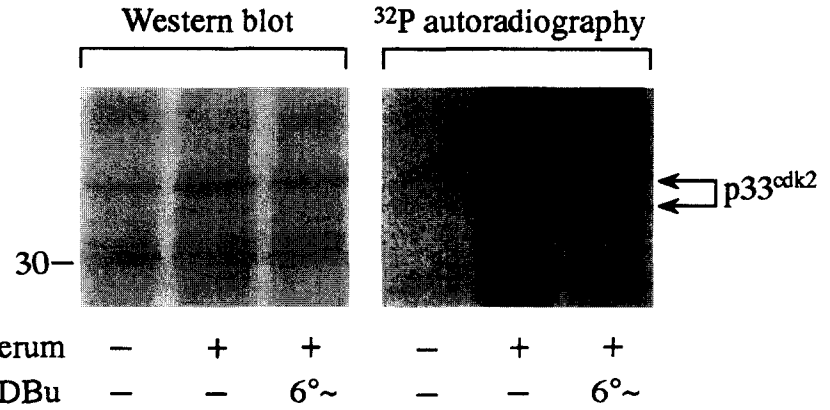

Fig. 3. Western blot and autoradiogram of CDK2 immunoprecipitated from ^{32}P-labeled cells. Quiescent cells were incubated in the presence or absence of serum and PDBu for a total of 20 h as indicated. During the last 4 h cells were metabolically labeled with [^{32}P]orthophosphate in phosphate-free medium. CDK2 was immunoprecipitated, separated on 12% SDS-PAGE and transferred to a membrane. After Western blot analysis with anti-CDK2 antibody, the membrane was exposed to a Fuji BAS 2000 IP plate and radioactivity detected.

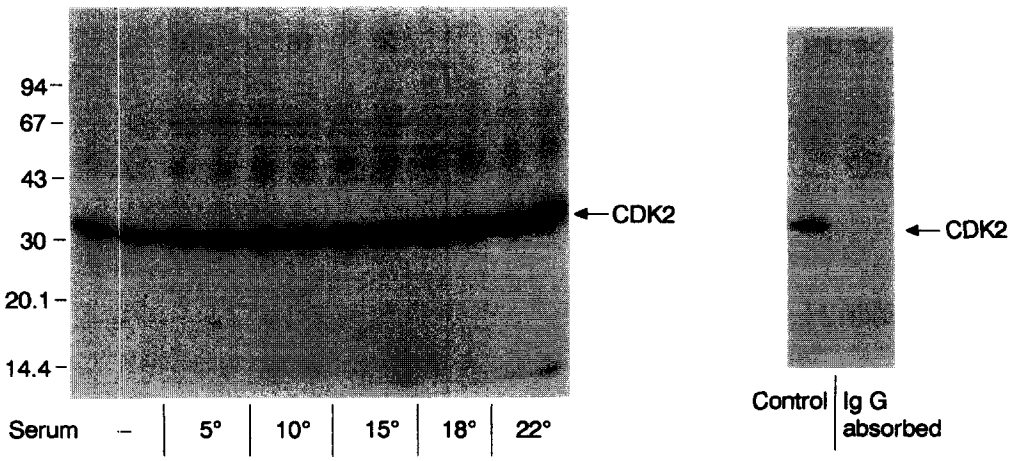

Fig. 4. Left: time-dependent changes in the CAK kinase activity in serum-stimulated IMR-90 cells. Quiescent cells were incubated with 10% fetal calf serum for indicated periods of time. CAK was then immunoprecipitated from 230 μg of cellular protein and the kinase activity was measured in vitro with recombinant human CDK2 as a substrate. The position of phosphorylated CDK2 is indicated on the right. Numbers on the left, molecular mass in kDa. Right: CAK kinase activity is not recovered when the antibody has been immunoabsorbed by preincubation with the antigen peptide.

phosphorylation of CDK2 at Thr160 causes increased mobility on SDS-PAGE, and that substitution of Thr160 to Ala abolishes the CAK-mediated phosphorylation and the mobility shift on SDS-PAGE [32]. In fact, serum stimulation causes stimulation of ^{32}P-labelling of the faster migrating band of CDK2 (Fig. 3). Therefore, the second band with a higher mobility most likely represents Thr160-phosphorylated form of CDK2. The addition of PDBu 6 h after serum stimulation results in complete disappearance of the second band on immunoblot and a marked reduction in the ^{32}P radioactivity incorporated into this band, without a detectable change in the amount of the major 33 kDa band on immunoblot (Fig. 3). The results indicate that activating phosphorylation of CDK2 at Thr160 [16–18] is suppressed by PKC activation. In addition, Fig. 3 also shows that serum stimulation leads to increased phosphorylation of CDK2 at sites other than Thr160, as demonstrated by phosphorylation of the major 33 kDa band, and that PKC activation inhibits this process as well.

Since it has now been established that phosphorylation of CDK2 at Thr160 is specifically mediated by CAK [16–18], the results suggest that PKC stimulation leads to inhibition of the CAK activity. To evaluate this possibility, we raised a polyclonal antibody against CDK7, the catalytic subunit of CAK, and studied changes in the CAK kinase activity in response to serum stimulation and PDBu treatment in IMR-90 cells. Shown in Fig. 4 (left) are changes in the CAK activity in serum-stimulated IMR-90 cells. Quiescent cells show a moderate level of basal CAK activity, as reported previously for other cell lines [33,34]. Differently from them, however, serum stimulation of IMR-90 cells results in a moderate, sustained increase in

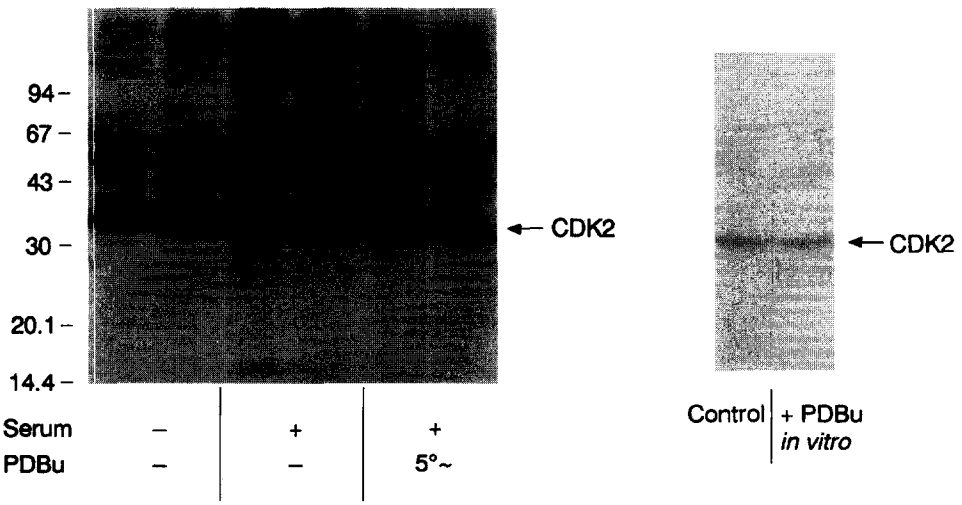

Fig. 5. Left: addition of PDBu 5 h after serum stimulation inhibits serum-induced increase in the CAK kinase activity. Quiescent cells were incubated with or without serum and PDBu (10^{-7} M) for a total of 18 h, and then the CAK activity was measured. Right: PDBu does not inhibit the CAK kinase activity when directly introduced into a kinase assay tube.

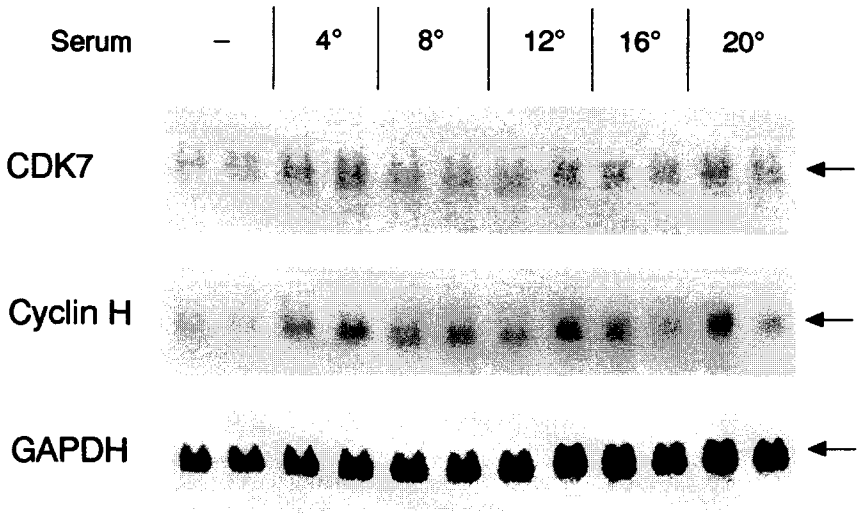

Fig. 6. Northern blot analysis of changes in the mRNA levels of CDK7 and cyclin H, the catalytic and the regulatory subunit of CAK, respectively. Quiescent cells were stimulated with serum for indicated time periods (h) and 10 μg each of total cellular mRNA was analyzed. GAPDH was adopted as an internal control.

the CAK activity. Thus, the CAK activity starts to increase within 5 h of serum stimulation, when 1.3-fold stimulation over the basal level is observed. The CAK activity reaches the plateau value of approximately 1.5-fold over the basal level by 10 h, which is sustained for up to 22 h of observations. Preincubation of the anti-CDK7 antibody (IgG fraction) with the antigen peptide completely abolishes the CAK kinase activity (Fig. 4, right). A sustained increase in the CAK activity after serum stimulation of quiescent cells has also been reported very recently for Swiss 3T3 fibroblasts [35]. As shown in Fig. 5 (left), if PDBu (10^{-7} M) is added 5 h after serum stimulation, it inhibits serum-stimulated increase in the CAK activity down to the basal level. Thus, the CAK activity 18 h after serum stimulation without PDBu treatment is $148 \pm 6\%$ (mean $\pm$ S.E. of four determinations; $P < 0.01$ as compared with quiescent cells by Students' t-test), whereas the CAK activity in serum-stimulated, PDBu-treated cells ($97 \pm 3\%$ of the basal level in quiescent cells) is not significantly different from that in quiescent cells. The addition of PDBu directly to kinase assay tubes has no effect on the CAK activity in vitro (Fig. 5, right). Very recently, it has

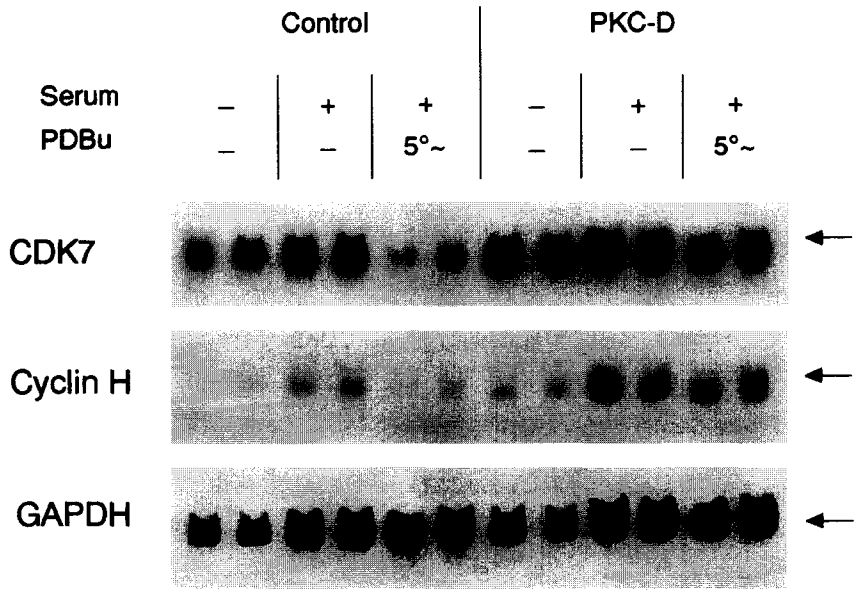

Fig. 7. Addition of PDBu 5 h after serum stimulation potently inhibits increases in the mRNA levels of both CDK7 and cyclin H in control, but not PKC-down-regulated (PKC-D), IMR – 90 cells. Cells were preincubated in DMEM/BSA containing either 0.1% dimethylsulfoxide (control) or 1 μM PDBu (PKC-D) for 48 h and at the same time made quiescent. Cells were then extensively washed and further incubated in the presence or absence of serum and PDBu (10^{-7} M) for 22 h. GAPDH was adopted as an internal control.

been demonstrated that CAK (CDK7/cyclin H complex) is associated with a general transcription factor IIH (TFIIH) and is responsible for the TFIIH-associated kinase activity that phosphorylates an essential carboxyl terminal domain of the RNA polymerase II large subunit, which is a critical step that occurs during initiation of transcription [36–39]. It is also shown that MO15 is directly involved in TFIIH-associated nucleotide excision repair activity [36]. Indeed, CAK activity is reported to be recovered as two major peaks with different mobilities after gel filtration procedure [17]. It is intriguing to speculate that PDBu treatment of IMR-90 cells specifically down-regulates a subpopulation of CAK that is responsible for phosphorylation of CDK family kinases, without affecting a subpopulation that associates with TFIIH. In fact, in spite of the complete cell cycle arrest, serum-induced increases in the cellular protein content are barely affected by PDBu treatment in IMR-90 cells, suggesting specific inhibitory effects of PDBu on the cell cycle machinery but not on housekeeping functions. Further studies are required to elucidate this point.

We further studied the effects of serum and PDBu on the mRNA level of CDK7 and cyclin H, the catalytic and the regulatory subunits of CAK, respectively (Figs. 6 and 7). In serum-stimulated IMR-90 cells, the message level of both CDK7 and cyclin H nearly doubles over the basal quiescent level by 4 h, and stays at this level for up to 20 h (Fig. 6). The mRNA level of GAPDH, adopted as an internal control, shows an 1.2-fold increase only after 12 h, and reaches the maximal level of 1.5-fold increase after 20 h. The addition of PDBu 5 h after serum stimulation completely cancels out the stimulatory effects of serum on both CDK7 and cyclin H mRNA levels, but not that of GAPDH (Fig. 7). The selective inhibition by PDBu treatment on both CDK7 and cyclin H mRNA levels are markedly attenuated in PKC-down-regulated cells, suggesting that this inhibition is mediated by a down-regulation-sensitive PKC isoform(s). These results indicate that PKC activation in the late G1 phase of serum-stimulated cells leads to inhibition of the CAK activity to the basal unstimulated level, at least in part through suppression of serum-induced increases in the mRNA level of both the catalytic and the regulatory subunits of CAK.

We have previously shown in human umbilical vein endothelial cells that PKC-mediated inhibition of CDK2 activation is associated with complete suppression of increases in the mRNA level of the partner cyclin molecules, cyclins A and E, but not CDK2 itself [8,28]. The same set of changes are also observed in IMR-90 cells (data not shown). The results strongly suggest that molecular mechanisms underlying PKC-mediated cell cycle arrest are similar, if not identical, among different cell types. CAK phosphorylates CDK2 in vitro equally well whether or not cyclin A is present [17]. However, activation of CDK2 as a protein kinase absolutely requires complex formation with a partner cyclin molecule in addition to Thr160 phosphorylation [16,17]. Although the exact sequence of in vivo activation events of CDK2 (first cyclin binding and then Thr160 phosphorylation or vice versa) is not well understood, the present study provides compelling evidence that two steps of CDK2 activation, cyclin A induction and CAK-mediated phosphorylation of CDK2, are both completely suppressed by PKC activation in the late G1 phase, a time frame when the two events of the CDK2 activation process are close to, or are being turned on. It is possible, however, that PDBu-mediated down-regulation of cyclin A mRNA level is the result of PKC-mediated cell cycle arrest. With this respect, it is of interest to test whether PKC activation in the late G1 phase causes any increase in the level of a CDK inhibitor. Recently it was shown in a macrophage cell line that activation of protein kinase A leads to cell cycle arrest through induction of the CDK inhibitor p^{27Kipl}, which binds to CDK4 and inhibits CAK-mediated phosphorylation of CDK4 in the face of normal CAK activity [40]. However, we failed to detect any increase in the protein level of p^{27Kipl} upon PDBu treatment. We are now under investigation regarding potential involvement of other CDK inhibitors in PKC-mediated G1 arrest.

Our results strongly suggest that down-regulation-sensitive isoform(s) of PKC mediates the inhibitory effects of PDBu on the CAK-CDK2 cascade. In studies with human umbilical vein endothelial cells, we have found that PKC-α, -δ, -ε and ζ are present in this cell type and that the first three isoforms are sensitive to down-regulation procedure [28]. However, the same set of PKC isoforms are present in Swiss 3T3 fibroblasts, in which PDBu induces activation of CDC2 and initiation of DNA synthesis [41]. Therefore it remains unknown at present which isoform(s) is responsible for PDBu-induced cell cycle arrest in endothelial cells and IMR-90 fibroblasts. Further studies are required to elucidate molecular mechanism(s) of PKC-mediated cell cycle regulation, which involves modulation of the message level of molecules constituting the cell cycle machinery.

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